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Use of glycoside hydrolase family 8 xylanases in baking

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Abstract

Xylanases have long been used in the baking industry for improving dough stability and flexibility and for increasing bread volume and crumb structure. Only xylanases from glycoside hydrolase families 10 and 11 appear to have been tested in this application and only those from the latter family have as yet found application. Interestingly, enzymes with a putative xylanase activity are also found in glycoside hydrolase families 5, 7, 8 and 43, but apparently these have not, as yet, been tested in baking. Baking trials were used to determine the effectiveness of a psychrophilic and a mesophilic family 8 xylanolytic enzyme as well as a psychrophilic family 10 xylanase and a currently used family 11 commercial mesophilic xylanase. The potential of family 8 xylanases as technological aids in baking was clearly demonstrated as both the psychrophilic enzyme from *Pseudoalteromonas haloplanktis* TAH3a and the mesophilic enzyme from *Bacillus halodurans* C-125 had a positive effect on loaf volume. In contrast, the psychrophilic family 10 enzyme from *Cryptococcus adeliae* TAE85 was found to be ineffective.

Keywords: Xylanase; Baking; Glycoside hydrolase family 8

1. Introduction

Xylanases are widely used as additives in the baking industry to improve processing and product quality. They have been shown to effect enhancements in dough and bread quality leading to improved dough flexibility, machinability and stability and a larger loaf volume as well as an improved crumb structure (Baillet et al., 2003; Guy and Sarabjit, 2003; Maat et al., 1992; Qi Si and Drost-Lustenberger, 2002). First introduced to the baking industry in the 1970s they are currently used frequently in combination with amylases, lipases and various oxidoreductases where specific effects on the rheological properties of the dough and organoleptic properties of the bread are desired (Qi Si and Drost-Lustenberger, 2002).

While the exact mechanism of the functionality of xylanases in breadmaking has not as yet been fully elucidated, it is currently believed that the redistribution of water from the arabinoxylan in the flour to the starch and gluten phases is important (Ingelbrecht et al., 2000; Rouau et al., 1994). Flour generally consists of approximately 80% starch and 12% proteins, with arabinoxylan content varying from 2–3% in wheat flour (Baillet et al., 2003), up to 5% in wholemeal wheat flour and 8% in rye flour. The arabinoxylan, even though present in minor amounts, is an extremely important functional ingredient as it can bind almost ten times its own weight in water, accounting for almost 30% of the water binding capacity of wheat flour and hence exerts a significant effect on the flour and accordingly the dough and bread quality (Baillet et al., 2003; Courtin and Delcour, 2002). Arabinoxylans are complex polymers composed of a (1→4)-β-D-xylopyranosyl backbone chain substituted with α-L-arabinofuranose residues at the C(O)2 and/or C(O)3 positions which may themselves be further linked to glucuronic acid residues and/or ferulic acid groups (Fincher and Stone, 1986; Rubio, 2003). Arabinoxylans in wheat flour typically consist of approximately 20–25% of a water-extractable fraction which has a strong influence on the viscosity of the aqueous medium and a water-unextractable fraction which has an extremely strong water holding capacity and a negative effect on dough quality, being detrimental to

Abbreviations: GH, glycoside hydrolase family; GH11 Xyl, family 11 mesophilic xylanase from *Bacillus subtilis*; IU, international units of xylanase activity; pXyl, glycoside hydrolase family 8 psychrophilic xylanase from *Pseudoalteromonas haloplanktis* TAH3a; Rex, glycoside hydrolase family 8 mesophilic enzyme from *Bacillus halodurans* C-125; X_B, glycoside hydrolase family 10 psychrophilic xylanase from *Cryptococcus adeliae* TAE85.

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the formation of the protein films in the dough due to physical interruption (Gruppen et al., 1992; Meuser and Suckow, 1986; Wang et al., 2003). The insoluble nature of this latter is due to interactions, both covalent and non-covalent, with adjacent arabinoxylan, protein and/or cellulose molecules (Vardakou et al., 2003b).

Due to differences in their substrate specificities, action patterns, interactions with inhibitors and kinetic capabilities, not all xylanases are effective in baking. Indeed, it is currently generally accepted that those most suited for breadmaking are those active on the water-unextractable fraction and poorly active on the water-extractable fraction, solubilising the insoluble arabinoxylan to give high molecular weight solubilised arabinoxylans and hence leading to a net loss of water holding capacity and an increased viscosity (Courtin et al., 1999; Rouau et al., 1994; Sorensen, 2003; Sorensen et al., 2004; Vardakou et al., 2003a). This action not only removes the insoluble arabinoxylan which interferes with the formation of the gluten network in dough but also increases the stability of the dough system due to the increased viscosity. This in turn yields a more stable and flexible dough which is easier to handle and gives improved oven spring, a larger loaf volume, improved crumb structure (fine crumbs and thin gas cell walls) and hence also a softer crumb (Courtin et al., 1999; Qi Si and Drost-Lustenberger, 2002; Sorensen, 2003).

Presently, it appears that only GH10 and 11 xylanases have been tested in baking applications and beneficial effects have been reported with members of the latter family only. Interestingly, enzymes with a putative xylanase activity have also been reported in glycoside hydrolase families 5, 7, 8 and 43 (Collins et al., 2005) but apparently these have not as yet been tested in baking. Here, the utility of GH8 enzymes in this application was investigated. Baking trials were performed with the cold-adapted GH8 xylanase (pXyl) from *Pseudoalteromonas haloplanktis* TAH3a (Collins et al., 2002a, 2003; Van Petegem et al., 2002, 2003) as well as the mesophilic GH8 enzyme (Rex) from *Bacillus halodurans* C-125 (Fushinobu et al., 2005; Honda and Kitaoka, 2004; Takami et al., 2000), the cold-adapted GH10 xylanase (X_B) from *Cryptococcus adeliae* TAE85 (Petrescu et al., 2000) and a commercial mesophilic GH11 xylanase (GH11 Xyl) from *Bacillus subtilis* to determine the effectiveness of these enzymes as baking additives.

2. Experimental

2.1. Family 8 psychrophilic xylanase (pXyl) from Pseudoatteromonas haloplanktis *TAH3a*

Production and purification of this recombinant enzyme was performed as described previously (Collins et al., 2002a). Briefly, cultures were produced in Terrific broth (12 g/l Bacto tryptone (Difco, USA), 24 g/l yeast extract (Difco, USA), 4 ml/l glycerol, 12.54 g/l K_2HPO_4, 2.31 g/l KH_2PO_4, 200 μg/ml ampicillin) at 18 °C, enzyme expression was induced with 1 mM isopropyl-1-thio-β-galactopyranoside and purification performed using a combination of anion exchange (QFF-sepharose, Amersham Biosciences, Sweden), cation exchange (SFF-sepharose, Amersham Biosciences, Sweden) and gel filtration (Sephacryl S-100, Amersham Biosciences, Sweden) chromatographies.

2.2. Family 10 psychrophilic xylanase (X_B) from Cryptococcus adeliae *TAE85*

Wild-type X_B from *C. adeliae* TAE85 was produced and purified using a procedure similar to that already described (Petrescu et al., 2000). Briefly, the yeast was grown for 7 d at 4 °C in a modified minimal medium (Petrescu et al., 2000), the xylanase precipitated by bringing the culture filtrate to 80% w/v ammonium sulphate and the enzyme purified by anion exchange chromatography on a Q-Sepharose Fast Flow column (Amersham Biosciences, Sweden).

2.3. Family 8 mesophilic Rex from Bacillus halodurans C-125

The gene sequence for Rex from *B. halodurans* C-125 was obtained from the Genbank/GenPept™ database (accession code BA000004, gene BH2105) and the wild-type organism (LMG 7121/ATCC BAA-125) was obtained from the BCCM™/LMG bacteria collection.

Genomic DNA was extracted and purified from 16 h cultures grown at 37 °C in modified Alkaline Bacillus medium (10 g/l glucose, 5 g/l yeast extract (Difco, USA), 10 g/l Bacto Tryptone (Difco, USA), 1 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 10 g/l Na_2CO_3, 10 g/l birchwood xylan (Sigma-Aldrich, USA)) with the Wizard® Genomic DNA Purification kit (Promega, USA). The entire Rex gene was PCR-amplified using VENT polymerase (Biolabs Inc, USA) with the sense primer 5′-GGGCATATGAAGAAAACGACAGAAGGTG-3′, containing an Nde I site (underlined) and the antisense primer 5′-GGCTCGAGCTAGTGTTCCTCTTCTTG-3′, containing an *Xho* I site (underlined) and the stop codon (in italics). The PCR product was cloned into a PCRScript Amp SK(+) vector (Stratagene, USA), sequenced on an ALF DNA sequencer (Pharmacia Biotech, Sweden) then excised with *Nde* I and *Xho* I and ligated into the pET 22b(+) cloning vector (Novagen, USA). The resulting recombinant plasmid was transformed to *E. coli* BL21 (DE3) cells (Stratagene, USA) for production and purification.

The cells from a 5 h preculture (37 °C) of *E. coli* BL21(DE3) carrying the Rex gene were collected by centrifugation at 10,000 g for 1 min and used to inoculate 5 l (15 ml preculture per liter of culture) of Terrific broth (12 g/l Bacto tryptone (Difco, USA), 24 g/l yeast extract (Difco, USA), 4 ml/l glycerol, 12.54 g/l K_2HPO_4, 2.31 g/l KH_2PO_4) containing 200 μg/ml ampicillin. The culture was incubated at 37 °C and 250 rpm until an absorbance at 550 nm of between 3 and 4 was reached whereupon the expression of the enzyme was induced with 1 mM isopropyl-1-thio-β-galactopyranoside. After 4 h further incubation at 37 °C the cells were harvested by centrifugation at 18,000 g for 20 min at 4 °C, resuspended in 20 mM MOPS (Sigma-Aldrich, USA), disrupted in a prechilled

cell disrupter (Constant Systems Ltd, UK) at 28 Kpsi and centrifuged at 40,000 *g* for 30 min. Chromosomal DNA was removed by treatment with 0.2% protamine sulfate (Calbiochem, USA) and 25 units of benzonase (Merck, Germany) followed by centrifugation at 40,000 *g* for 30 min. This crude extract was used in the baking trials.

2.4. Family 11 mesophilic xylanase (GH11 Xyl) from Bacillus subtilis

The family 11 mesophilic xylanase from *Bacillus subtilis* which is sold for baking applications under the product name Bel'ase B210 was provided by Beldem Puratos, Andenne, Belgium.

2.5. Baking trials

The effectiveness of the various enzyme preparations was evaluated in mini-baking trials, in the production of Belgian hard rolls and in the preparation of Argentinean long breads. All baking trials were performed in duplicate.

2.5.1. Mini-baking trials

Flour (Surbi Molens van Deinze), 100 g; water, 57 g; yeast (Bruggeman-Belgium), 5 g; sodium chloride, 2 g; dextrose, 2 g; ascorbic acid, 4 g and various concentrations of the individual enzyme preparations were mixed for 4.5 min in a National mixer (USA). Dough pieces (150 g) were weighed and rested for 20 min at 25 °C in plastic boxes, reworked and then rested for another 20 min. The doughs were then proofed at 36 °C for 50 min, baked at 225 °C for 20 min and the volume of the loaves produced was determined by the rapeseed displacement method.

2.5.2. Belgian hard rolls

Flour (Surbi Molens van Deinze), 1500 g; water, 915 g; yeast (Bruggeman), 90 g; sodium chloride, 30 g; Multec Data HP20 emulsifier (Beldem S.A., Belgium), 3.9 g; ascorbic acid, 0.12 g and various concentrations of the psychrophilic GH8 xylanase pXyl were mixed for 2 min at low and 8 min at high speed in a Diosna SP24 mixer (Diosna, Germany). The final dough resting and proofing temperature was 25 °C. After resting for 15 min the dough was reworked manually and rested for another 10 min. Dough pieces (2 kg) were made up and proofed for 10 min and then divided and moulded into 66 g round pieces using an Eberhardt Optimat moulder (Eberhardt, Germany). After another 5 min resting, the dough pieces were cut by pressing and submitted to a final proofing of 70 or 120 min. The dough pieces were baked at 230 °C in a MIWE Condo™ steam oven (Michael Wenz-Arnstein, Germany) and the volume of 20 rolls was measured for each enzyme preparation using the rapeseed displacement method.

2.5.3. Argentinean long breads

Flour (Surbi Molens van Deinze), 1500 g; water, 810 g; yeast (Bruggeman), 5.25 g; sodium chloride, 30 g; standard improver (210 units/g; Puratos), 22.5 g and various concentrations of the psychrophilic GH8 xylanase or the mesophilic GH11 xylanase were mixed for 2 min at low and 7 min at high speed in a Diosna SP24 mixer (Diosna, Germany). Final dough resting and proofing temperatures of 25 and 20 °C, respectively, were used throughout. After resting for 20 min at 25 °C, the dough was reworked manually and proofed for 17 h at 20 °C. Dough pieces (2 kg) were then made up and proofed for another 10 min after which 0.35 kg dough pieces were prepared using a Bertrand R8/L8 moulder (Bertrand, France) and submitted to a final proofing stage for 17 h. The dough pieces were baked at 210 °C for 30 min in a MIWE Condo™ steam oven (Michael Wenz-Arnstein, Germany) and the volume of six loaves was measured for each enzyme preparation using the rapeseed displacement method.

2.6. Enzyme activity measurements

Xylanase activity was measured by the Nelson–Somogyi method with 0.125% birchwood xylan (Sigma-Aldrich, USA) at 30 °C in 0.1 M sodium-acetate buffer at pH 4.5 (Nelson, 1944; Teleman et al., 2002). One international unit (IU) is defined as the amount of enzyme required to liberate 1 μmole of reducing sugar per minute. Protease activity was measured by incubating 100 μl of 0.5 M Tris, pH 8.0, 100 μl of 3% azocasein and 300 μl of a suitably diluted test solution at 25 °C for 10 min, stopping the reaction with 500 μl of 10% TCA, centrifuging at 15,000 *g* for 3 min and measuring the absorbance at 366 nm.

3. Results and discussion

In the present study, two psychrophilic xylanases from glycoside hydrolase families 8 and 10 were prepared, purified and tested for their effectiveness in baking applications. Both enzymes were purified to homogeneity as indicated by SDS-PAGE and were found to be free of protease activity. While addition of the psychrophilic GH8 enzyme, pXyl, to the baking mix in the mini-baking trials led to an increase in bread loaf volume as compared to a negative control without added xylanase (approximately 6 and 17% increase for 0.25 and 0.5 IU per kg of flour, respectively), use of the psychrophilic GH10 enzyme from *C. adeliae* TAE85 (X_B) had no significant effects (Table 1). Indeed, as can be seen in Figs. 1 and 2, increasing concentrations of pXyl in the preparation of Belgian hard rolls leads to an increased loaf volume, reaching saturation at approximately 2.0 IU per kg of flour. Furthermore, the use of this enzyme in the preparation of Argentinean long breads, where a long proofing time (17 h) at 20 °C is included, also leads to an increase in loaf volume as well as an increased cut-width, even though the amount of enzyme required to optimize each of these parameters is different i.e. 0.5 and 0.25, respectively (Fig. 3 and Table 2).

In addition to the psychrophilic GH8 xylanase, the effectiveness of a GH8 mesophilic enzyme was also tested. The gene encoding the mesophilic GH8 enzyme Rex was isolated and gene sequencing confirmed that it was identical to

EXHIBIT B

Table 1
Effect of the commercial family 11 xylanase from *B. subtilis* (GH11 Xyl), the psychrophilic family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl), the mesophilic family 8 enzyme from *B. halodurans* C-125 (Rex) and the psychrophilic family 10 xylanase from *Cryptococcus adeliae* TAE85 (X_B) on the final volume of bread loaves in the mini-baking trials

Xylanase	IU Xylanase/kg flour	Loaf volume (ml)
None–control	0	650
GH11 Xyl	6.3	680
GH11 Xyl	10.5	705
GH8 pXyl	0.25	690
GH8 pXyl	0.50	760
GH8 Rex	60	725
GH8 Rex	120	725
GH10 X_B	30	660
GH10 X_B	60	670

The error on the volume measurement using the rapeseed displacement method is approximately 1%. IU, International Units of xylanase activity; GH, glycoside hydrolase family.

that of gene BH2105 (Genbank/GenPept™ accession code BA000004). The recombinant enzyme was produced in *E. coli* and analysis by the Nelson–Somogyi method indicated the recombinant enzyme to be active on birchwood xylan under the conditions used. As in the case of the psychrophilic enzyme, use of this GH8 enzyme in mini-baking trials also led to an increase in bread loaf volume (Table 1), albeit apparently much less efficiently (as indicated by the high concentration of enzyme required).

A commercial GH11 enzyme from *Bacillus subtilis* (GH11 Xyl) was used as a positive control in all baking trials and, as expected, had a positive effect on the loaf volume. Indeed, all currently used industrial baking xylanases appear to belong to GH11 (see Table 3) while GH10 xylanases have been found to be ineffective in this application. In agreement with this, we showed the effectiveness of the mesophilic GH11 enzyme from *Bacillus subtilis* and the ineffectiveness of the psychrophilic GH10 enzyme from *C. adeliae* TAE85. More importantly, our study also clearly demonstrates the potential of GH8 enzymes

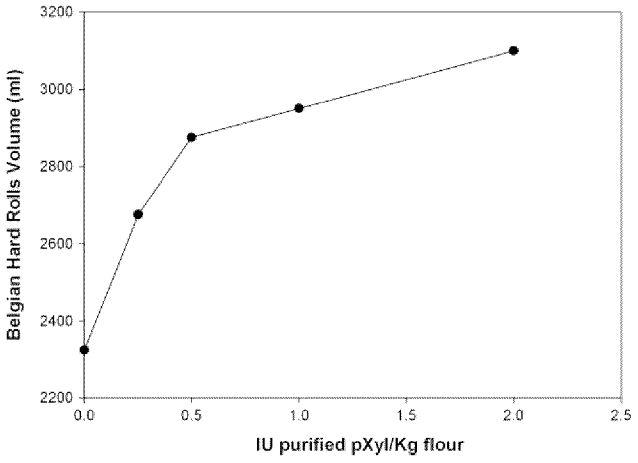

Fig. 1. Effect of the concentration of the psychrophilic family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl) on the volume of Belgian hard loaves. Proofing was carried out at 25 °C for 80 min. The error on the volume measurement using the rapeseed displacement method is approximately 1 %. IU, International Units of xylanase activity.

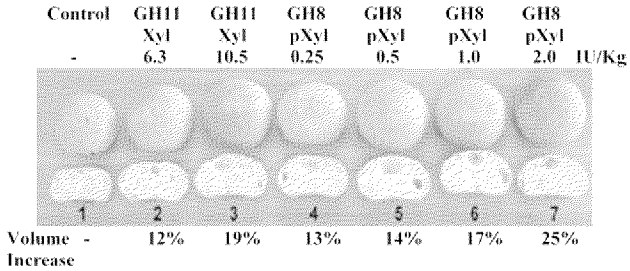

Fig. 2. Pictorial showing the effect of various concentrations of the commercial family 11 xylanase from *B. subtilis* (GH11 Xyl), loaves 2 and 3, and of the psychrophilic family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl), loaves 4–7, on the volume of Belgian hard rolls. The values below the pictorial indicate the % volume increase as compared to the control without xylanase, loaf 1. Proofing was carried out at 25 °C for 130 min. The error on the volume measurement using the rapeseed displacement method is approximately 1 %. IU/kg, International Units of xylanase activity per kilogram of flour.

as technological aids in baking since both the mesophilic and psychrophilic GH8 enzymes tested were found to be effective.

GH8 xylanases are a relatively recent discovery and the demonstration of their potential for use in baking applications will certainly increase interest in this family of enzymes and extend the repertoire of xylanases available for this application. Members of this glycoside hydrolase family display a distorted $(\alpha/\alpha)_6$ barrel fold (Fushinobu et al., 2005; Van Petegem et al., 2003), catalyse hydrolysis with inversion of the anomeric configuration at the newly exposed anomeric centre giving rise to products with an α-configuration (Collins et al., 2002a) and most probably use a glutamate and an aspartate as catalytic residues (Honda and Kitaoka, 2004). In contrast, GH10 and 11

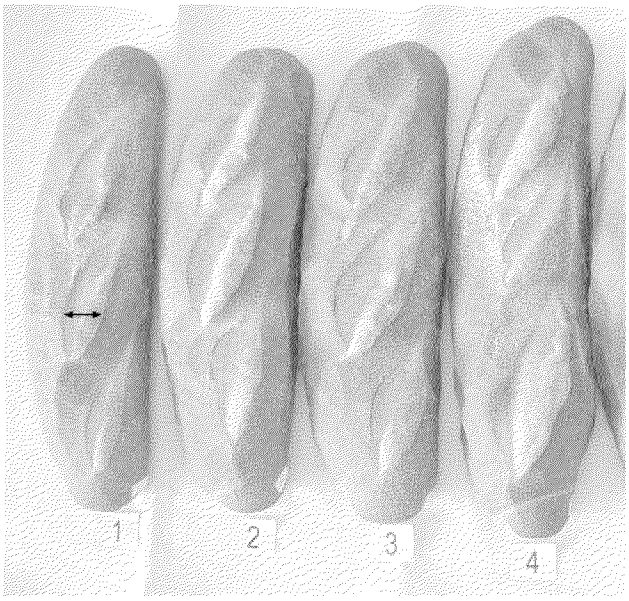

Fig. 3. Pictorial showing the effect of 21 IU of the commercial family 11 xylanase from *B. subtilis* (loaf 2) and 0.25 (loaf 3) or 0.5 (loaf 4) IU of the psychrophilic family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a per kilogram of flour on the volume and cut width (↔) of Argentinean long breads as compared to a negative control without added xylanases (loaf 1). See Table 2 for further details. The error on the volume measurement using the rapeseed displacement method is approximately 1 %.

Table 2
Comparison of the effect of the commercial family 11 xylanase from *B. subtilis* (GH11 Xyl), the psychrophilic family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl) and a negative control on the final volume and cut-widths of Argentinean long bread loaves

Xylanase	IU Xylanase/kg flour	Loaf volume (ml)	Width of cut (mm)
None–Control	0	1675	19
GH11 Xyl	21	1900	27
GH 8 pXyl	0.25	1900	34
GH 8 pXyl	0.50	1925	30

The error on the volume measurement using the rapeseed displacement method is approximately 1%. IU, International Units of xylanase activity; GH, glycoside hydrolase family.

xylanases display an $(\alpha/\beta)_8$ barrel or β-jelly roll fold, respectively, and catalyse hydrolysis with retention of the anomeric configuration, with two glutamate residues acting as the essential catalytic residues (Collins et al., 2005).

With respect to the effectiveness of xylanases in baking, the properties of most importance are probably the substrate specificity and action pattern (Vardakou et al., 2003a) as well as the effect of endogenous inhibitors present in the bread mix (Gebruers et al., 2004, 2005; Juge et al., 2004; Payan et al., 2004; Trogh et al., 2004). While the effect of endogenous inhibitors has not yet been studied, initial investigations of GH8 xylanases suggest these to have a similar substrate specificity (Brennan et al., 2004; Collins et al., 2002a) and action pattern to GH11 enzymes. They have a lower catalytic versatility than GH10 xylanases, being more hindered by substituents on the (1→4)-β-D-xylopyranosyl backbone chain and producing larger hydrolysis products (Biely et al., 1993, 1997). GH10 enzymes degrade xylan to a greater degree (Biely et al., 1993, 1997) and indeed their use in baking often leads to undesirable sticky doughs and a loss of water holding capacity due to the over-hydrolysis of both soluble and insoluble arabinoxylan (Rouau et al., 1994; Sorensen et al., 2004).

Table 3
Examples of some of the more commonly used xylanase preparations for the baking industry

Enzyme producer	Xylanase preparation	Wild-type host organism	Glycoside hydrolase family
AB enzymes	Veron® 191	*Aspergillus niger*	11
	Veron® Special	*Bacillus subtilis*	11
Beldem-Puratos	Bel'ase B210	*Bacillus subtilis*	11
	Bel'ase F25	*Aspergillus niger*	11
Danisco	Grindamyl™ H	*Aspergillus niger*	11
	Grindamyl™-Powerbake	*Bacillus subtilis*	11
DSM	Bakezyme® HS	*Aspergillus niger*	11
	Bakezyme® BXP	*Bacillus subtilis*	11
Genencor Intl.	Multifect® xylanase	*Trichoderma reesei*	11
	Multifect® XL	*Trichoderma reesei*	11
Novozymes	Pentopan Mono	*Thermomyces lanuginosus*	11

An interesting property of the GH8 enzyme pXyl is its psychrophilic character; this enzyme has an apparent optimal temperature of 35 °C (5 min assay) and retains approximately 90 and 60% of its maximum activity at 20 and 5 °C, respectively (Collins et al., 2003). In contrast, its mesophilic homologue, Rex, is optimally active at 50 °C (10 min assay) and retains only 20% of its maximum activity at 20 °C (Honda and Kitaoka, 2004). Thus the inherent high activity of the psychrophilic enzyme at the temperatures used for dough preparation and proofing may be one of the determinative factors in its observed high efficiency. Indeed, psychrophilic enzymes may be highly suited for use in the baking industry as they are generally optimally active at the temperatures (Collins et al., 2002b) most frequently used for dough preparation and proofing (around or below 35 °C) and could offer more efficient baking additives than the currently used commercial mesophilic enzymes which are generally optimally active at higher temperatures.

In summary, the potential for use of GH8 xylanolytic enzymes and, in particular, the psychrophilic xylanase pXyl as technological aids in baking has been demonstrated for the first time. The potential for psychrophilic enzymes, in general, in the baking industry has also been evoked, not only for GH8 psychrophilic xylanases, but also potentially for psychrophilic GH5, 7, 11 and 43 xylanases (Collins et al., 2005) as well as psychrophilic proteases, lipases, oxidoreductases, etc.

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